REMARKS

Attached hereto is a marked-up version of the changes made to the specification by the current Preliminary Amendment. The attached page is captions "Version with markings to show changes made".

Respectfully submitted,

Dated: May 10, 2001

Ronald B. Hildreth
Patent Office Reg. No. 19,498

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Version With Markings to Show Changes Made

This application is a continuation of copending International application PCT/IL99/00483 filed September 6, 1999, which is incorporated by reference herein, claiming priority from Israeli application Nos. 126112 filed September 7, 1998, and 126757 filed October 26,1998. The International application was published in English on March 16, 2000, by the International Bureau .

VERSION*

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C12N 15/63, 15/67, 15/11, 15/85, 5/10, C07K 14/525, A01K 67/027, A61K 48/00

A1

(11) International Publication Number: WO 00/14255

(43) International Publication Date: 16 March 2000 (16.03.00)

(21) International Application Number: PCT/IL99/00483

(22) International Filing Date: 6 September 1999 (06.09.99)

(30) Priority Data:

126112	7 September 1998 (07.09.98)	IL
126757	26 October 1998 (26.10.98)	IL

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

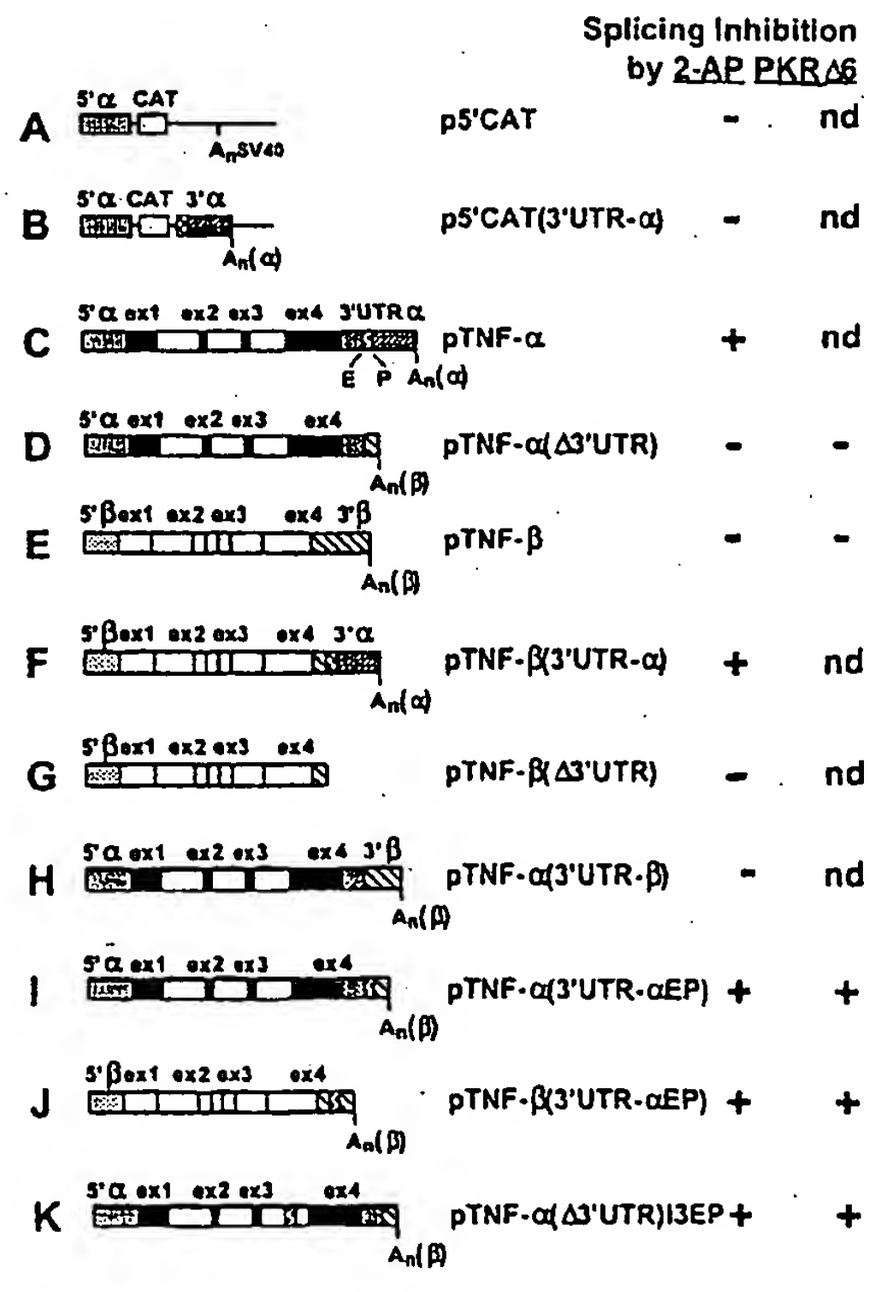
With a revised version of the international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the revised version of the international search report: 4 May 2000 (04.05.00)

(54) Title: REGULATION OF GENE EXPRESSION THROUGH MANIPULATION OF mRNA SPLICING AND ITS USES

(57) Abstract

A *cis*-acting nucleotide sequence which is capable of rendering the removal of intron/s from a precursor transcript encoded by a gene, which gene harbors at least one such *cis*-acting nucleotide sequence, occurring during the production of mRNA of said gene, dependent upon activation of a *trans*-acting factor. The *trans*-acting factor is an RNA-activated protein kinase which is capable of phosphorylating the α-subunit of eukaryotic initiation factor 2, or the RNA-activated protein kinase (PKR). The *cis*-acting nucleotide sequence can be derived from the 3' untranslated region of the human tumor necrosis factor α gene (TNF-α 3'-UTR). The *cis*-acting nucleotide sequence may comprise the nucleotide sequence substantially as denoted by SEQ ID NO:1; or biologically functional fragments, derivatives, mutants and homologues of the nucleotide sequence substantially as denoted by SEQ ID NO:1; or a nucleotide sequence whose complementary nucleotide sequence hybridizes, under conditions which allow for such hybridization to occur, with the nucleotide sequences substantially as denoted by SEQ ID NO:1 or biologically functional fragments, derivatives, mutants and homologues of the nucleotide sequence substantially as denoted by SEQ ID NO:1.



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INTERNATIONAL SEARCH REPORT

Inte ..ional Application No
PCT/IL 99/00483

A CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/63 C12N15/67 C12N15/11 C12N15/85 C12N5/10
C07K14/525 A01K67/027 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A01K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	OSMAN F (REPRINT) ET AL: "A cis-acting element in the 3'-UTR of human TNF-alpha mRNA renders splicing dependent on activation of protein kinase PKR" EUROPEAN CYTOKINE NETWORK, (SEP 1998) VOL. 9, NO. 3, PP. 479-479. PUBLISHER: JOHN LIBBEY EUROTEXT LTD, 127 AVE DE LA REPUBLIQUE, 92120 MONTROUGE, FRANCE. , page 324, XP000867413 Abstract no. 479 abstract --- -/--	1-31

[X] Further documents are listed in the continuation of box C

[X] Patent family members are listed in annex

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 January 2000	04/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P B 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx 31 651 epo nl, Fax: (+31-70) 340-3016	Hornig, H

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. tional Application No
PCT/IL 99/00483

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate of the relevant passages	Relevant to claim No
A	N. JARROUS ET AL.: "2-Aminopurine selectively inhibits splicing of tumor necrosis factor alpha mRNA" MOL. CELL. BIOL., vol. 16, no. 6, June 1996 (1996-06), pages 2814-2822, XP002128326 ASM WASHINGTON, DC,US cited in the application the whole document	
A	WO 94 21661 A (UNIV LELAND STANFORD JUNIOR) 29 September 1994 (1994-09-29) the whole document	
A	S. DAVIS AND J.C. WATSON: "In vitro activation of the interferon-induced, double-stranded RNA-dependent protein kinase PKR by RNA from the 3' untranslated regions of human alpha-tropomyosin" PROC. NATL. ACAD. SCI., vol. 93, January 1996 (1996-01), pages 508-513, XP002128327 NATL. ACAD. SCI.,WASHINGTON,DC,US; cited in the application the whole document	
A	J. WANG AND J.L: MANLEY: "Regulation of pre-mRNA splicing in metazoa" CURRENT OPINION IN GENETICS & DEVELOPMENT, vol. 7, no. 2, April 1997 (1997-04), pages 205-211, XP000867380 CURRENT BIOLOGY LTD., PHILADELPHIA, US page 209, left-hand column, line 18 -right-hand column, line 44	
A	WO 97 27309 A (AGRONOMIQUE INST NAT RECH ;ELOIT MARC (FR); ADAM MICHELINE (FR)) 31 July 1997 (1997-07-31) the whole document	
A	EP 0 309 237 A (GENENTECH INC) 29 March 1989 (1989-03-29) the whole document	
A	GROSKREUTZ D ET AL: "TRANSIENT EXPRESSION: INCREASED GENE EXPRESSION IN MAMMALIAN CELL LINES USING PADVANTAGE(TM) DNA AS CO-TRANSFECTANT" PROMEGA NOTES,XX,XX, vol. 48, page 8-12 XP002014330 the whole document	
A	US 5 559 019 A (GUI JIAN-FANG ET AL) 24 September 1996 (1996-09-24) the whole document	

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INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/IL 99/00483

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication,where appropriate of the relevant passages	Relevant to claim No
T	F. OSMAN ET AL.: "A cis-acting element in the 3'-untranslated region of human TNF-alpha mRNA renders splicing dependent on the activation of protein kinase PKR" GENES & DEVELOPMENT, vol. 13, no. 24, 15 December 1999 (1999-12-15), pages 3280-3293, XP002128328 CSH LABORATORY PRESS, NEW YORK,US the whole document	1-31, 34-42

INTERNATIONAL SEARCH REPORT

International application No
PCT/IL 99/00483

Box I Observations where certain claims were found unsearchabl (Continuation of item 1 of first sh et)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1 [X] Claims Nos :
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 43 and 44 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2 [ ] Claims Nos :
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically

3 [ ] Claims Nos :
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6 4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4 [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. .tional Application No
PCT/IL 99/00483

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9421661 A	29-09-1994	AU 6490894 A	11-10-1994
		EP 0690869 A	10-01-1996
WO 9727309 A	31-07-1997	FR 2743818 A	25-07-1997
		AU 1447997 A	20-08-1997
EP 0309237 A	29-03-1989	US 5024939 A	18-06-1991
		AT 111156 T	15-09-1994
		CA 1340480 A	06-04-1999
		DE 3851399 D	13-10-1994
		DE 3851399 T	20-04-1995
		ES 2061671 T	16-12-1994
		JP 1165395 A	29-06-1989
		JP 2738544 B	08-04-1998
US 5559019 A	24-09-1996	NONE	

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 7310-7311/WO/99	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/IL99/00483	International filing date *(day/month/year)* 06/09/1999	Priority date *(day/month/year)* 07/09/1998
International Patent Classification (IPC) or national classification and IPC C12N15/63		
Applicant YISSUM RESEARCH DEVELOPMENT COMPANY OF THE..et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 30 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
02/04/2000	05.01.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Marinoni, J-C Telephone No. +49 89 2399 8563

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IL99/00483

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1,2,4,5,9,11-14, 20-45	as originally filed				
3,6-8,10,15-19	as received on	13/10/2000	with letter of	10/10/2000	

Claims, No.:

1-54	as received on	13/10/2000	with letter of	10/10/2000

Drawings, sheets:

1/14-14/14	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

☐ the description, pages:

☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	16, 19-24, 26, 30, 36, 37 and 41-54
	No:	Claims	1-15, 17, 18, 25, 27-29, 31-35, 38 and 39
Inventive step (IS)	Yes:	Claims	NONE
	No:	Claims	1-54
Industrial applicability (IA)	Yes:	Claims	1-54
	No:	Claims	NONE

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following documents:

D1: D. PENNICA ET AL.: 'Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin' NATURE, vol. 312, 20/27 December 1984, pages 724-729

D2: N. JARROUS ET AL.: '2-Aminopurine selectively inhibits splicing of tumor necrosis factor alpha mRNA' MOL. CELL. BIOL., vol. 16, no. 6, June 1996, pages 2814-2822 cited in the application

D1 was not cited in the international search report. A copy of the document is appended hereto.

2. The subject-matter of **claims 1-15, 17, 18, 25, 27-29, 31-35, 38 and 39** is not new in the sense of Article 33(2) PCT.

D1 discloses the nucleotide and amino acid sequences of the human TNF-α gene, including SEQ. ID NO: 1 and 2 of the present application (see page 725, figure 1, nucleotides 1069-1173, and 1073-1116). Following the objection raised under **item VIII-1**, it is concluded that **D1** discloses the sequence (*i.e.* the native gene) and a DNA construct having the inherent features claimed in **claims 1-15, 17, 18 and 25** of the present application.

D2 discloses a vector (phTNF-α) comprising the TNF-α gene, including the 3' untranslated region, a carrier (salmon sperm DNA) and a host cell transformed with said vector wherein said cell is from the baby hamster kidney (BHK-21) cell line (see page 2815 column 1, lines 7, 17-21 and 25) as in **claims 27-29 and 31-35** of the present application.
D2 also discloses a method of regulating gene expression at the mRNA level transforming a host cell with the above mentioned vector wherein the activity of the RNA activated eIF2αkinase in said host cell is modulated by the use of 2-Aminopurine (see page 2820, column 1, lines 5-24) as in **claims 38 and 39** of the

present application.

Hence, **claims 1-15, 17, 18, 25, 27-29, 31-35, 38 and 39** are not new in the sense of Article 33 (2) PCT.

3. The subject-matter of **claims 16, 19-24, 26, 30, 36, 37, 41-54** does not involve an inventive step in the sense of Article 33 (3) PCT.

D2, which is considered to represent the closest prior art discloses, as indicated above, a method of regulating gene expression at the mRNA level transforming a host cell with the above mentioned vector wherein the activity of the RNA activated eIF2α kinase in said host cell is modulated by the use of 2-Aminopurine (see page 2820, column 1, lines 5-24).
Furthermore, **D2** clearly indicates that "Most likely, regulation by 2-AP is mediated through a particular sequence within the TNF-α primary transcript to produce general inhibition of the splicing of this transcript." (see page 2821 column 1, lines 38-40) and that "..deletion of a particular sequence from the TNF-α gene renders splicing of the encoded precursor transcripts resistant to inhibition by 2-AP, while introduction of said sequence into the TNF-β gene shifts the inhibitory effect of 2-AP on the TNF-β gene expression from transcription to splicing" is showed by the authors (see page 2821 column 1, lines 45-51). Taken in combination this two sentences make it clear that the sequence of interest in comprised within the TNF-α transcript (*i.e.* not in any other region of the gene).
Moreover **D2** states that "These findings strengthen the concept emerging from studies on IL-1ß and IL-2 gene expression that the rate of splicing of precursor RNA is tightly regulated and serves as a limiting step in expression of cytokine mRNA. The sensitivity of splicing of TNF-α precursor transcripts to 2-AP can serve as a valuable tool for further study of this type of post-transcriptional control".

The difference between **D2** and the present application lies in the fact that in **D2** the exact location of the particular sequence within the TNF-α primary transcript that mediates the general inhibition of the splicing of this transcript and of other genes in which it may be introduced is not disclosed.

The problem to be solved may therefore be regarded as how to define the exact location of said sequence.

The Applicant solves the problem by exactly locating said sequence through routine molecular genetics techniques.

Due to the clear teachings of **D2**, in order to solve the problem posed, the person skilled in the art would have been prompted to locate said sequence, would have exactly located it and would hence, have used said sequence for post-transcriptional regulation studies and applications.

Hence, the subject-matter of **claims claims 16, 19-24, 26, 30, 36, 37, 41-54** does not fulfill the requirements of Article 33(3) PCT.

Re Item VIII
Certain observations on the international application

1. The subject-matter of **claim 1** is defined in terms of the result to be achieved (see the Guidelines Ch. III, 4.7) but not in terms of positive technical features (see Rule 6 PCT) that could allow a clear characterization of the intended sequence and thereby allow to distinguish it from similar subject-matters of the prior art.
   The same objection applies *mutatis mutandis* to **claim 9**.

2. The terms "homologue" and "derivatives" used in **claims 4-7 and 10-13** is vague and unclear and leaves the reader in doubt as to the meaning of the technical features to which it refers, thereby rendering the definition of the subject-matter of said claims unclear (Article 6 PCT).
   The same objection applies to the expression "under conditions that allows for such hybridisation to occur" in **claims 5, 7, 11 and 13**.

3. **Claims 19, 20, 23, 24 and 26** refer to figures. Such is however not allowed (see the Guidelines, Ch. III, 4.10).

4. **Claim 28** appears to refer back incorrectly to claim 23 thereby introducing doubt as to the claimed subject-matter (Article 6 PCT).

5. **Claims 47-50** refer to medical treatments. The Applicant is requested to note that the present wording of said claims may not be acceptable upon to entry into the regional phase; in fact, the patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment under Article 52(4) EPC, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

This invention deals with another major level of control of gene expression which has received little attention up to now: mRNA splicing, which is the processing of precursor transcripts into mature mRNA containing only exon sequences, by excision of introns at the RNA level in the cell nucleus. The mRNA splicing step is a good candidate for control since evidence exists that this mechanism functions *in vivo* [12, 13]. There are several examples of genes requiring a splicing event for mRNA production [24] and an intron generally is included in pharmaceutically employed expression vectors [5, 6]. For complementary DNA (cDNA) expression, the contribution of the intron to final product formation seems to be cDNA-specific but the mechanism of intron action remains largely unknown [25]. To date, little effort has been directed at regulation expression of genes for biotechnological use or gene therapy at the mRNA splicing step. Regulation of mRNA splicing would be useful for regulating expression of genes that have been transferred, be it into cell lines, the germline or somatic tissues.

Expression of several cytokine genes is highly regulated at splicing of precursor transcripts [12, 13, 27-29]. Thus, shortly after the onset of induction of human interleukin-2 (IL-2) and interleukin-1β (IL-1β) genes, the flow of nuclear precursor transcripts into mature mRNA becomes blocked despite the fact that transcription, once activated by an inducer, continues unabated for an extensive period. Expression of IL-2 and IL-1β mRNA is superinduced by two orders of magnitude in the presence of translation inhibitors, without a significant increase in primary transcription or mRNA stability. Instead, splicing of precursor transcripts is greatly facilitated [13, 27].

The cDNA and predicted amino acid sequences of human tumor necrosis factor-α (TNF-α) are described, for example, in Pennica, D. et al., Nature 312(20/27): 724-729. Expression of the TNF-α gene is also regulated at splicing [13]. 2-Aminopurine (2-AP) blocks expression of TNF-α mRNA in primary human lymphoid cells. An adenine isomer, 2-AP inhibits specific kinases that phosphorylate the α-subunit of eukaryotic translation initiation factor 2 (eIF2α) [17], including the RNA-activated protein kinase, PKR [30]. 2-AP does not inhibit human TNF-α gene expression at transcription, nor does it affect mRNA stability. Instead, splicing of short-lived TNF-α precursor transcripts into mRNA is blocked when 2-AP

Summary of the Invention

The present invention relates to *cis*-acting nucleic acid sequences which render the removal of intron/s from a precursor transcript encoded by a gene which contains at least one such *cis*-acting nucleic acid sequence, which occurs during the production of mRNA of the gene, dependent upon activation of a *trans*-acting factor which is an RNA-activated protein kinase capable of phosphorylating the α-subunit of eukaryotic initiation factor 2 (eIF2α).

In specific embodiments the RNA-activated protein kinase is the RNA-activated protein kinase (PKR).

In a preferred embodiment the *cis*-acting nucleic acid sequence of the invention is derived from the 3' untranslated region of the human tumor necrosis factor α gene (TNF-α 3'-UTR).

In especially preferred embodiments the *cis*-acting nucleic acid sequence of the invention comprises the nucleotide sequence as denoted by SEQ ID NO:1. The invention also relates to biologically functional fragments, derivatives, mutants and homologues of this sequence. The invention further relates to nucleotide sequences which can hybridize with complementary nucleotide sequences of SEQ ID NO:1 and of the biologically functional fragments, derivatives, mutants and homologues thereof.

In a most preferred embodiment the *cis*-acting nucleic acid sequence of the invention comprises SEQ ID NO:2 and biologically functional fragments, derivatives, mutants and homologues thereof.

SEQ ID NO:1 and SEQ ID NO:2 are provided, hereinafter, in Table 1.

The *cis*-acting nucleic acid sequences according to the invention are capable of rendering the removal of intron/s from a precursor transcript encoded by a gene which harbors at least one such *cis*-acting nucleic acid sequence, which occurs during the production of mRNA of the gene, dependent upon activation of a *trans*-acting factor which is an RNA-activated protein kinase capable of phosphorylating the α-subunit of

eukaryotic initiation factor 2 (eIF2α). The gene can be any gene of interest, including genes having a therapeutic, industrial, agricultural or any other commercial value or genes encoding proteins which are of therapeutic, industrial, agricultural or of any other commercial value.

In a further aspect, the invention relates to a DNA construct comprising a gene which contains at least one intron; a *cis*-acting nucleotide sequence which is capable of rendering the removal of intron/s from a precursor transcript encoded by said gene, which gene includes at least one such *cis*-acting nucleotide sequence, occurring during the production of mRNA of said gene, dependent upon activation of a *trans*-acting factor, said *trans*-acting factor being an RNA-activated protein kinase which is capable of phosphorylating the α-subunit of eukaryotic initiation factor 2, operably linked to said gene; and optionally further comprising additional control, promoting and/or regulatory elements.

In particular embodiments, said *cis*-acting nucleotide sequence in the DNA construct according to the invention comprises the nucleotide sequence as denoted by SEQ ID NO:1 or by SEQ ID NO:2, or biologically functional fragments, derivatives, mutants and homologues of the nucleotide sequence as denoted by SEQ ID NO:1 or by SEQ ID NO:2.

The invention relates in a further particular embodiment to a nucleotide sequence whose complementary sequence hybridizes, under conditions which allow for such hybridization to occur, with the said nucleotide sequences.

In the DNA constructs according to the invention, said gene is preferably a gene which encodes a protein is selected from the group consisting of enzymes, hormones, growth factors, cytokines, structural proteins and industrially or agriculturally applicable proteins, or is itself a therapeutic product, an agricultural product, or an industrially applicable product.

In the DNA constructs according to the invention said nucleotide sequence can be contained within an exon or within an intron of said gene.

In a further aspect, the invention relates to a vector comprising the *cis*-acting nucleotide sequence or a DNA construct according to the invention and a suitable DNA carrier, capable of transfecting a host cell with said *cis*-acting nucleotide sequence.

In an additional aspect, the invention relates to a host cell transfected with a *cis*-acting nucleotide sequence or with a DNA construct according to the invention, capable of expressing substantial amounts of said gene. The transfected host cells in accordance with the invention are preferably eukaryotic or yeast cells.

In addition, the invention relates to a non-human transgenic animal carrying in its genome a *cis*-acting nucleotide sequence or a DNA construct according to the invention, which is capable of expressing substantial amounts of said gene.

Additionally, the invention relates to a transgenic plant carrying in its genome a *cis*-acting nucleotide sequence or a DNA construct according to the invention, which is capable of expressing substantial amounts of said gene.

Furthermore, the invention relates to methods of regulating gene expression at the mRNA splicing level by (a) providing a *cis*-acting nucleotide sequence which is capable of rendering the removal of intron/s from a precursor transcript encoded by a gene which contains at least one intron dependent upon activation of a *trans*-acting factor, said *trans*-acting factor being an RNA-activated protein kinase which is capable of phosphorylating the α-subunit of eukaryotic initiation factor 2; (b) operably linking said *cis*-acting nucleotide sequence to said gene to give a DNA construct; (c) optionally combining the construct thus obtained with a suitable DNA carrier and optionally operably linking the same to suitable additional expression control, promoting and/or regulatory elements to give a transfection vector which is capable of transfecting a host cell; (d) transfecting a host cell with a *cis*-acting nucleotide sequence of the invention, or with a DNA construct of the invention or with the transfection vector, wherein the host cell is capable of expressing an RNA-activated protein kinase which is capable of phosphorylating the α-subunit of eukaryotic

Brief Description of the Drawings

Figure 1A-1K *Gene constructs used*

Introns are white boxes; ex, exon; 3'UTRα, TNF-α 3'-UTR; A_n, polyadenylation and cleavage site of TNF-α gene (α), TNF-β gene (β) or SV40; nd, not determined; 5'α, 3'α and 3'β are defined in the text. E, EcoRI site; P, PstI site.

Figure 2A-2F *TNF-α 3'-UTR sequences are needed for inhibition of mRNA splicing by 2-AP*

BHK-21 cells were transfected with pTNF-α **(A, B-D)**, p5'αCAT **(E)** or pTNF-α(Δ3'UTR) DNA **(F)** and co-transfected with pSV_2CAT DNA **(B, F)**. 2-AP was present from time of transfection at the concentrations shown **(A)**, or from 18 h thereafter at 10 mM **(B-F)**. Cell viability remained constant. Total RNA was isolated at times shown after transfection and subjected to RNase protection analysis with ^{32}P-labeled TNF-α antisense RNA probes P **(A, B, F)** to quantitate correctly initiated TNF-α RNA **(A**: 169-nt band), TNF-α RNA carrying a correct 3' end **(A**: 83-nt band), TNF-α precursor transcripts **(B, F**: 700-nt band) or spliced RNA **(B, F**: 341-nt band). In **(B)**, upper autoradiogram shows a higher exposure of 700-nt band. Autoradiogram of **(B)** was subjected to densitometry; intensity of 700-nt band **(C)** and 341-nt band **(D)**, expressed in the absence (○, □) or presence of 2-AP (●,■), is plotted. In B, E and F, CAT mRNA protects 250 nt of probe. In A and E, α-actin probe detects a 215-nt portion of mRNA. In autoradiograms A and E, left lane shows untransfected cells and in F, cells transfected with pSV_2CAT DNA alone.

Figure 3A-3E *TNF-α 3'-UTR sequences suffice to confer splicing control by 2-AP*

BHK-21 cells were transfected with pTNF-β **(A, B)**. pTNF-β(3'UTR-α) **(C, D)** or pTNF-α(3'UTR-β) DNA **(E)** and

which allow for such hybridization to occur, with the nucleotide sequences of SEQ ID NO:1.

A most preferred *cis*-acting nucleotide sequence according to the invention comprises (a) the nucleotide sequence as denoted by SEQ ID NO:2; or (b) biologically functional fragments, derivatives, mutants and homologues of the nucleotide sequence as denoted by SEQ ID NO:2.

Another preferred *cis*-acting nucleotide sequence according to the invention comprises a nucleotide sequence whose complementary nucleotide sequence hybridizes, under conditions which allow for such hybridization to occur, with the nucleotide sequences of SEQ ID NO:2.

SEQ ID NO:1 and SEQ ID NO:2 are shown in the following Table 1.

Table 1

SEQ ID NO:1

```
    GAATTCAAACTGGGGCCTCCAGAACTCACTGGGGCCTACAGCTTTGATCCCTGACATCTG
2817---------+---------+---------+---------+---------+----------+2876
    CTTAAGTTTGACCCCGGAGGTCTTGAGTGACCCCGGATGTCGAAACTAGGGACTGTAGAC

    GAATCTGGAGACCAGGGAGCCTTTGGTTCTGGCCAGAATGCTGC
2877---------+---------+---------+---------+----2920
    CTTAGACCTCTGGTCCCTCGGAAACCAAGACCGGTCTTACGACG
```

SEQ ID NO:2

```
    TCAAACTGGGGCCTCCAGAACTCACTGGGGCCTACAGCTTTGA
2821-----+---------+---------+---------+-------2863
    CTTAAGTTTGACCCCGGAGGTCTTGAGTGACCCCGGATGTCGA
```

As shown in Example 7 and Fig. 5B, 3'UTR-αEP RNA forms a stable, 5'-proximal 48-nt stem-loop containing 17 base pairs (DG= -59 kJ at 30°C).The DNA encoding this stem loop is denoted herein by SEQ ID NO:2.

The term "functional" as used herein is to be understood as any such sequence which would render the removal of introns from precursor mRNA transcripts encoded by a gene which harbors such sequences, dependent upon activation of a *trans*-acting factor which is an RNA-activated protein kinase capable of phosphorylating eIF2α.

In a further aspect the invention relates to a DNA construct comprising a gene which contains at least one intron; a *cis*-acting nucleotide sequence which is capable of rendering the removal of intron/s from a precursor transcript encoded by said gene, which gene includes at least one such *cis*-acting nucleotide sequence, occurring during the production of mRNA of said gene, dependent upon activation of a *trans*-acting factor, said *trans*-acting factor being an RNA-activated protein kinase which is capable of phosphorylating the α-subunit of eukaryotic initiation factor 2, operably linked to said gene; and optionally further comprises additional control, promoting and/or regulatory elements.

The control, promoting and/or regulatory elements are suitable transcription promoters, transcription enhancers and mRNA destabilizing elements, or any other suitable elements known to those skilled in the art.

In a specific embodiment, the *cis*-acting nucleotide sequence contained within a DNA construct of the invention comprises the nucleotide sequence as denoted by SEQ ID NO:1; or biologically functional fragments, derivatives, mutants and homologues of the nucleotide sequence as denoted by SEQ ID NO:1.

In another specific embodiment, the *cis*-acting nucleotide sequence contained within a DNA construct of the invention comprises a nucleotide sequence whose complementary sequence hybridizes, under conditions which allow for such hybridization to occur, with the nucleotide sequences denoted by SEQ ID NO:1 or with the biologically functional fragments, derivatives, mutants and homologues thereof.

In a particularly preferred embodiment the *cis*-acting nucleotide sequence contained within the DNA construct of the invention comprises the nucleotide sequence as

denoted by SEQ ID NO:2; or biologically functional fragments, derivatives, mutants and homologues of the nucleotide sequence as denoted by SEQ ID NO:2.

In another particularly preferred embodiment the *cis*-acting nucleotide sequence contained within the DNA construct of the invention comprises a nucleotide sequence whose complementary sequence hybridizes, under conditions which allow for such hybridization to occur, with the nucleotide sequence denoted by SEQ ID NO:2 or with the biologically functional fragments, derivatives, mutants and homologues thereof.

The *cis*-acting nucleotide sequence comprised in the DNA constructs of the invention may be contained within an exon or within an intron of the gene.

In the DNA construct of the invention said gene may encode a protein selected from the group consisting of enzymes, hormones, growth factors, cytokines, structural proteins and industrially or agriculturally applicable proteins, or the gene is itself a therapeutic product, an agricultural product, or an industrially applicable.

Specific DNA constructs according to the invention are such in which the gene is the human TNF-α gene. Examples for such constructs are the plasmid pTNF-α (Fig. 1C) and the plasmid pTNF-α(3'UTR-αEP) (Fig. 1I), in both of which the *cis*-acting element is contained within an exon of the gene. The *cis*-acting element may also be contained within an intron of said gene, as in, for example, the plasmid pTNF-α(Δ3'UTR)i3EP (Fig. 1K).

Other specific DNA constructs are such in which the gene is the human TNF-β gene. Examples for these constructs are the plasmid pTNF-β(3'UTR-α) (Fig. 1F) and the plasmid pTNF-β(3'UTR-αEP) (Fig. 1J), in both of which the *cis*-acting element is contained within an exon of the gene.

In a further aspect, the invention relates to a transfection vector comprising the *cis*-acting element of the invention, or functional fragments, derivatives, homologues or mutants thereof, optionally operably linked to suitable additional control, promoting and/or regulatory sequences. The vectors of the invention are designed to

facilitate the introduction of the *cis*-acting element into a host cell.

The vectors may contain suitable additional control, promoting and/or regulatory sequences of cellular and/or viral origin. The invention relates also to host cells transfected with a gene of interest operably linked to the *cis*-acting element of the invention, or with the *cis*-acting element of the invention itself, and to their various uses.

Specifically, the invention relates to a host cell transfected with a DNA construct or an expression vector according to the invention. Alternatively, the host cell may be transfected with DNA encoding a *cis*-acting element according to the invention itself. Host cells according to the invention can also be cells only transfected with the *cis*-acting nucleotide sequence of the invention.

The host cells according to the invention may be eukaryotic or yeast cells. Examples of eukaryotic cells are, *inter alia*, mammalian hemopoietic cells, fibroblasts, epithelial cells, or lymphocytes.

Specific host cells which may be transfected are the baby hamster kidney (BHK-21) cell line or the Chinese hamster ovary (CHO) cell line.

With the development of gene transfer techniques that allow the generation of transgenic animals came the possibility of producing animal bioreactors as an alternative strategy to cell culture systems for protein production [25]. For instance, protein secretion in the milk of large mammals could provide a cost effective route for the production of large amounts of valuable proteins. As yet this technology is still in development and needs optimization, and there is a general requirement for methods to improve productivity. The *cis*-acting nucleotide sequences according to the invention may help attain this goal by improved regulation of the expression of a desired protein.

Thus, the invention also relates to a non-human transgenic animal which carries in its genome a *cis*-acting nucleotide sequence or DNA construct in accordance with the

invention, which transgenic animal is capable of expressing substantial amounts of protein encoded by said gene.

The non-human transgenic animals of the invention may be used in a method of producing recombinant enzymes, hormones, growth factors, cytokines, structural proteins or other industrially or agriculturally applicable proteins, also encompassed by the present invention, which process comprises the steps of (a) providing a transgenic animal transformed with a DNA construct according to the invention, in which said gene encodes such enzyme, hormone, growth factor, cytokine, structural protein or another industrially or agriculturally applicable protein, said transgenic animal being capable of expressing said gene in substantial amounts; (b)growing the transgenic animal provided in (a) under suitable conditions to allow the said gene to be expressed; and (c) isolating the protein encoded by said gene from said animal, or from an egg or body secretion thereof. Techniques in which the gene is expressed, for example, in cattle's milk and chicken eggs may be used, and the desired protein encoded by the gene isolated.

Regulated expression could be achieved by several routes. To date, transcriptional regulation has received most attention [1-4], while little effort has been directed at improving efficiency of pre-mRNA processing. In the broader context, mechanisms allowing the regulation of RNA processing would assist gene transfer, be it into cell lines, the germline or somatic tissues. Transgenic animals, provide an appropriate model for testing gene therapy constructs, where an ability to regulate expression is of paramount importance.

The present discovery of the *cis*-acting element in the human TNF-α 3'-UTR that renders splicing of TNF-α mRNA sensitive to inhibition by 2-AP, provides a unique and novel tool for bringing expression of a desired gene under the control of this mechanism. Such regulation can be implemented by introducing the *cis*-acting element into expression vectors and generating cell lines in which the expression of PKR can be manipulated. The exonic *cis*-acting element from the TNF-α gene of this invention, is a portable element that confers splicing control. Since upon transport into the

CLAIMS:

1. A *cis*-acting nucleotide sequence which is capable of rendering the removal of intron/s from a precursor transcript encoded by a gene, which gene harbors at least one such *cis*-acting nucleotide sequence, occurring during the production of mRNA of said gene, dependent upon activation of a *trans*-acting factor, said *trans*-acting factor being an RNA-activated protein kinase which is capable of phosphorylating the α-subunit of eukaryotic initiation factor 2.

2. A *cis*-acting nucleotide sequence according to claim 1 wherein said *trans*-acting factor is the RNA-activated protein kinase (PKR).

3. A *cis*-acting nucleotide sequence according to claim 1 or claim 2 derived from the 3' untranslated region of the human tumor necrosis factor α gene (TNF-α 3'-UTR).

4. A *cis*-acting nucleotide sequence according to any one of claims 1 to 3 which comprises:

   a) the nucleotide sequence as denoted by SEQ ID NO:1; or

   b) functional fragments, derivatives, mutants and homologues of the nucleotide sequence as denoted by SEQ ID NO:1, that are capable of rendering the removal of intron/s from a precursor mRNA encoded by a gene, which gene harbors at least one such *cis*-acting nucleotide sequence, dependent upon activation of a *trans*-acting factor, said *trans*-acting factor being an RNA-activated protein kinase capable of phosphorylating eIF2α.

5. A *cis*-acting nucleotide sequence according to any one of claims 1 to 3 which comprises a nucleotide sequence whose complementary nucleotide sequence hybridizes, under conditions which allow for such hybridization to occur, with the nucleotide sequence as denoted by SEQ ID NO:1 or with the functional fragments, derivatives, mutants and homologues defined in claim 4.

6. A *cis*-acting nucleotide sequence according to any one of claims 4 and 5 which comprises:

a) the nucleotide sequence as denoted by SEQ ID NO:2; or

b) functional fragments, derivatives, mutants and homologues of the nucleotide sequence as denoted by SEQ ID NO:1, that are capable of rendering the removal of intron/s from a precursor mRNA encoded by a gene, which gene harbors at least one such *cis*-acting nucleotide sequence, dependent upon activation of a *trans*-acting factor, said *trans*-acting factor being an RNA-activated protein kinase capable of phosphorylating eIF2α.

7. A *cis*-acting nucleotide sequence according to any one of claims 1 to 3 and 6 which comprises a nucleotide sequence whose complementary nucleotide sequence hybridizes, under conditions which allow for such hybridization to occur, with the nucleotide sequences as denoted by SEQ ID NO:2 or with the functional fragments, derivatives, mutants and homologues defined in claim 6.

8. A *cis*-acting nucleotide sequence according to any one of claims 1 to 7 wherein said gene encodes a protein selected from the group consisting of enzymes, hormones, growth factors, cytokines, structural proteins and industrially or agriculturally applicable proteins, or is itself a therapeutic product, an agricultural product, or an industrially applicable product.

9. A DNA construct comprising:-

- a gene which contains at least one intron;
- a *cis*-acting nucleotide sequence which is capable of rendering the removal of intron/s from a precursor transcript encoded by said gene, which gene includes at least one such *cis*-acting nucleotide sequence, occurring during the production of mRNA of said gene, dependent upon activation of a *trans*-acting factor, said *trans*-acting factor being an RNA-activated protein kinase which is capable of phosphorylating the α-subunit of eukaryotic initiation factor 2, operably linked to said gene; and

- optionally further comprising additional control, promoting and/or regulatory elements.

10. A DNA construct according to claim 9 wherein said *cis*-acting nucleotide sequence comprises:

a) the nucleotide sequence as denoted by SEQ ID NO:1; or

b) functional fragments, derivatives, mutants and homologues of the nucleotide sequence as denoted by SEQ ID NO:1, that are capable of rendering the removal of intron/s from a precursor mRNA encoded by a gene, which gene harbors at least one such *cis*-acting nucleotide sequence, dependent upon activation of a *trans*-acting factor, said *trans*-acting factor being an RNA-activated protein kinase capable of phosphorylating eIF2α.

11. A DNA construct according to claims 9 wherein said *cis*-acting nucleotide sequence comprises a nucleotide sequence whose complementary sequence hybridizes, under conditions which allow for such hybridization to occur, with the nucleotide sequences as denoted by SEQ ID NO:1 or with the functional fragments, derivatives, mutants and homologues defined in claim 5.

12. A DNA construct according to claim 9 wherein said *cis*-acting nucleotide sequence comprises:

a) the nucleotide sequence as denoted by SEQ ID NO:2; or

b) functional fragments, derivatives, mutants and homologues of the nucleotide sequence as denoted by SEQ ID NO:2, that are capable of rendering the removal of intron/s from a precursor mRNA encoded by a gene, which gene harbors at least one such *cis*-acting nucleotide sequence, dependent upon activation of a *trans*-acting factor, said *trans*-acting factor being an RNA-activated protein kinase capable of phosphorylating eIF2α.

13. A DNA construct according to claims 12 wherein said *cis*-acting nucleotide sequence comprises a nucleotide sequence whose complementary sequence hybridizes, under conditions which allow for such hybridization to occur, with the nucleotide sequences as denoted by SEQ ID NO:2 or with the functional fragments, derivatives, mutants and homologues defined in claim 7.

14. A DNA construct according to any one of claims 9 to 13 wherein said control, promoting and/or regulatory elements are suitable transcription promoters, transcription enhancers and mRNA destabilizing elements.

15. A DNA construct according to claim 9 wherein said gene encodes a protein selected from the group consisting of enzymes, hormones, growth factors, cytokines, structural proteins and industrially or agriculturally applicable proteins, or is itself a therapeutic product, an agricultural product, or an industrially applicable product.

16. A DNA construct according to any one of claims 9 to 15 wherein said nucleotide sequence is contained within an exon of said gene.

17. A DNA construct according to any one of claims 9 to 15 wherein said nucleotide sequence is contained within an intron of said gene.

18. A DNA construct according to any one of claims 9 to 17 wherein said gene is the human TNF-α gene.

19. A DNA construct according to claim 18 being the plasmid pTNF-α as shown in Figure 1C, in which said *cis*-acting element is contained within an exon of the human TNF-α gene.

20. A DNA construct according to claim 19 being the plasmid pTNF-α(3'UTR-αEP), as shown in Figure 1I.

21. A DNA construct according to any one of claims 9 to 17 wherein said gene is

the human TNF-β gene.

22. A DNA construct according to claim 21 in which said *cis*-acting element is contained within an exon of the human TNF-β gene.

23. A DNA construct according to claim 22 being the plasmid pTNF-β(3'UTR-α) as shown in Figure 1F.

24. A DNA construct according to claim 22 being the plasmid pTNF-β(3'UTR-αEP), as shown in Figure 1J.

25. A DNA construct according to claim 18 in which said gene is the human TNF-α gene and said *cis*-acting element is contained within an intron of said gene.

26. A DNA construct according to claim 25 being the plasmid pTNFα(Δ3'UTR)i3EP, as shown in Figure 1K.

27. A vector comprising a *cis*-acting nucleotide sequence according to any one of claims 1 to 8 or a DNA construct according to any one of claims 9 to 26 and a suitable DNA carrier, capable of transfecting a host cell with said *cis*-acting nucleotide sequence.

28. A vector according to claim 23 optionally further comprising additional expression, control, promoting and/or regulatory elements operably linked thereto.

29. A vector according to claim 28 wherein said carrier is salmon sperm DNA.

30. A vector according to claim 28 wherein said carrier is viral DNA.

31. A host cell transfected with a DNA construct according to any one of claims 9 to 26.

32. A host cell transfected with a vector according to claim 27.

33. A host cell according to claim 31 or 32 being a eukaryotic or yeast cell.

34. A host cell according to claim 33 being a mammalian hemopoietic cell, fibroblast, epithelial cell, or lymphocyte.

35. A host cell according to claim 31 wherein said eukaryotic cell is the baby hamster kidney (BHK-21) cell line or the Chinese hamster ovary (CHO) cell line.

36. A non-human transgenic animal carrying in its genome a DNA construct according to any one of claims 9 to 26, said transgenic animal being capable of expressing substantial amounts of said gene.

37. A non-human transgenic animal transformed with an expression vector according to claim 27, said transgenic animal being capable of expressing substantial amounts of said gene.

38. A method of regulating gene expression at the mRNA splicing level comprising the steps of:

a) providing a *cis*-acting nucleotide sequence which is capable of rendering the removal of intron/s from a precursor transcript encoded by a gene which contains at least one intron dependent upon activation of a *trans*-acting factor, said *trans*-acting factor being an RNA-activated protein kinase which is capable of phosphorylating the α-subunit of eukaryotic initiation factor 2;

b) operably linking said *cis*-acting nucleotide sequence to said gene to give a DNA construct;

c) optionally linking to the construct obtained in step (b) additional expression control, promoting and/or regulatory elements to give an expression vector;

d) transforming a host cell with the DNA construct obtained in (b) or with the expression vector obtained in (c), said host cell being capable of

expressing an RNA-activated protein kinase which is capable of phosphorylating the α-subunit of eukaryotic initiation factor 2, to give a transformed host cell capable of expressing said gene in substantial amounts, wherein the expression and/or activity of the RNA-activated eIF2α kinase in said host cell is modulated.

39. A method according to claim 38 wherein the activity of the RNA-activated eIF2α kinase in said host cell is modulated by use of 2-aminopurine or other adenine derivatives.

40. A method according to claim 38 wherein the activation of the RNA-activated eIF2α kinase in said host cell is modulated by use of a transdominant negative mutant of PKRΔ6.

41. A method according to claim 38 wherein the activation of the RNA-activated eIF2α kinase in said host cell is chemically modulated.

42. A method according to claim 41 wherein said modulation is effected by Ca^{2-} ions.

43. A method according to claim 38 wherein the activity of the RNA-activated eIF2α kinase in said host cell is modulated by use of a vector expressing viral proteins.

44. A method according to claim 43 wherein said vector is vaccinia E3L protein or vaccinia K3L protein.

45. A method according to claim 38 wherein the activity of the RNA-activated eIF2α kinase in said host cell is modulated by use of a vector expressing viral RNA.

46. A method according to claim 45 wherein said vector is an adenovirus VA RNA or the Epstein-Barr virus Eber RNA.

47. A method for *ex vivo* treating an individual suffering an acquired or hereditary

pathological disorder in which a therapeutic product is not made by said individual, or made is in abnormally low amounts or in a defective form or is made in essentially normal amount to be increased comprising:

a) providing a DNA construct according to any one of claim 9 to 26 or an expression vector according to any one of claims 27 to 30 wherein said gene encodes said therapeutic product;

b) obtaining cells from an individual suffering said disorder and optionally culturing said cells under suitable conditions;

c) transfecting the cells obtained in (b) with a DNA construct or expression vector provided in (a); and

d) re-introducing said cells obtained in (c) into said individual.

48. A method of *ex vivo* treating an individual suffering from a pathological disorder requiring increase of expression of a therapeutic product normally made by said individual in physiological amount comprising:

a) providing DNA construct according to any one of claims 9 to 26 or an expression vector according to any one of claims 27 to 30, wherein said gene encodes said therapeutic product;

b) obtaining cells from an individual suffering said disorder and optionally culturing said cells under suitable conditions;

c) transfecting the cells obtained in (b) with a DNA construct or expression vector provided in (a); and

d) re-introducing said cells obtained in (c) into said individual.

49. A method of providing a therapeutic protein product to a mammal comprising administering to the mammal a DNA construct according to any one of claims 9 to 26, wherein said gene encodes said therapeutic protein product.

50. A method of providing a therapeutic protein product to a mammal comprising administering to the mammal a therapeutically effective amount of transformed

host cells according to any one of claims 31 to 35, wherein said gene encodes said therapeutic protein product.

51. A pharmaceutical composition comprising as active ingredient a therapeutically effective amount of expression vectors according to any one of claims 27 to 30 or of transformed host cells according to any one of claims 31 to 35.

52. A method of producing a recombinant therapeutic or industrially or agriculturally applicable protein comprising the steps of:

a) providing a DNA construct according to any one of claim 9 to 26 or an expression vector according to any one of claims 27 to 30 wherein said gene encodes said protein;

b) transfecting a host cell with a DNA construct or expression vector provided in (a) to give a host cell capable of expressing said protein in substantial amount; and

c) culturing cells obtained in (b) under suitable culture conditions; and

d) isolating said protein from the cell culture obtained in (c).

53. A method of producing a recombinant therapeutic or industrially or agriculturally applicable protein comprising the steps of:

a) providing host cells transfected with a DNA construct according to any one of claim 9 to 26 or an expression vector according to any one of claims 27 to 30 wherein said gene encodes said protein, which are capable of expressing said protein in substantial amount;

b) culturing cells provided in (a) under suitable culture conditions; and

c) isolating said protein from the cell culture obtained in (b).

54. A method of producing a recombinant enzyme, hormone, growth factor, cytokine, structural protein or another industrially or agriculturally applicable protein, comprising the steps of:-

a) providing a transgenic animal transformed with a DNA construct according to any one of claims 9 to 26, wherein said gene encodes an enzyme, a hormone, a growth factor, a cytokine, a structural protein or an industrially or agriculturally applicable protein, said transformed animal being capable of expressing said gene in substantial amounts;

b) growing the transgenic animal provided in (a) under suitable conditions to allow the said gene to be expressed; and

c) isolating the protein encoded by said gene from said animal, or from an egg or body secretion thereof.

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* 7310-7311/WO/99

Box No. I TITLE OF INVENTION

REGULATION OF GENE EXPRESSION THROUGH MANIPULATION OF mRNA SPLICING AND ITS USES

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State *(i.e. country)* of nationality: IL

State *(i.e. country)* of residence: IL

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address:

This person is:

☐ applicant only

☐ applicant and inventor

☐ inventor only

State *(i.e. country)* of nationality:

State *(i.e. country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on a continuation sheet

Box No. V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

- ☒ **AP** **ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SL Sierra Leone, SZ Swaziland, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☒ **EA** **Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☒ **EP** **European Patent:** AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☒ **OA** **OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* . . . . . .

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- ☒ AE United Arab Emirates . . . . . .
- ☒ AL Albania . . . . . .
- ☒ AM Armenia . . . . . .
- ☒ AT Austria . . . . . .
- ☒ AU Australia . . . . . .
- ☒ AZ Azerbaijan . . . . . .
- ☒ BA Bosnia and Herzegovina . . . . . .
- ☒ BB Barbados . . . . . .
- ☒ BG Bulgaria . . . . . .
- ☒ BR Brazil . . . . . .
- ☒ BY Belarus . . . . . .
- ☒ CA Canada . . . . . .
- ☒ CH and LI Switzerland and Liechtenstein . . . . . .
- ☒ CN China . . . . . .
- ☒ CU Cuba . . . . . .
- ☒ CZ Czech Republic . . . . . .
- ☒ DE Germany . . . . . .
- ☒ DK Denmark . . . . . .
- ☒ EE Estonia . . . . . .
- ☒ ES Spain . . . . . .
- ☒ FI Finland . . . . . .
- ☒ GB United Kingdom . . . . . .
- ☒ GD Grenada . . . . . .
- ☒ GE Georgia . . . . . .
- ☒ GH Ghana . . . . . .
- ☒ GM Gambia . . . . . .
- ☒ HR Croatia . . . . . .
- ☒ HU Hungary . . . . . .
- ☒ ID Indonesia . . . . . .
- ☒ IL Israel . . . . . .
- ☒ IN India . . . . . .
- ☒ IS Iceland . . . . . .
- ☒ JP Japan . . . . . .
- ☒ KE Kenya . . . . . .
- ☒ KG Kyrgyzstan . . . . . .
- ☒ KP Democratic People's Republic of Korea. . . .
- ☒ KR Republic of Korea . . . . . .
- ☒ KZ Kazakstan . . . . . .
- ☒ LC Saint Lucia . . . . . .
- ☒ LK Sri Lanka . . . . . .
- ☒ LR Liberia . . . . . .
- ☒ LS Lesotho . . . . . .
- ☒ LT Lithuania . . . . . .
- ☒ LU Luxembourg . . . . . .
- ☒ LV Latvia . . . . . .
- ☒ MD Republic of Moldova . . . . . .
- ☒ MG Madagascar . . . . . .
- ☒ MK The former Yugoslav Republic of Macedonia . . . . . .
- ☒ MN Mongolia . . . . . .
- ☒ MW Malawi . . . . . .
- ☒ MX Mexico . . . . . .
- ☒ NO Norway . . . . . .
- ☒ NZ New Zealand . . . . . .
- ☒ PL Poland . . . . . .
- ☒ PT Portugal . . . . . .
- ☒ RO Romania . . . . . .
- ☒ RU Russian Federation . . . . . .
- ☒ SD Sudan . . . . . .
- ☒ SE Sweden . . . . . .
- ☒ SG Singapore . . . . . .
- ☒ SI Slovenia . . . . . .
- ☒ SK Slovakia . . . . . .
- ☒ SL Sierra Leone. . . . . .
- ☒ TJ Tajikistan . . . . . .
- ☒ TM Turkmenistan . . . . . .
- ☒ TR Turkey . . . . . .
- ☒ TT Trinidad and Tobago . . . . . .
- ☒ UA Ukraine . . . . . .
- ☒ UG Uganda . . . . . .
- ☒ US United States of America . . . . . .
- ☒ UZ Uzbekistan . . . . . .
- ☒ VN Viet Nam . . . . . .
- ☒ YU Yugoslavia . . . . . .
- ☒ ZA South Africa . . . . . .
- ☒ ZW Zimbabwe . . . . . .

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:

- ☒ CR Costa Rica. . . . . .
- ☒ DM Dominica. . . . . .
- ☐ . . . . . .

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Supplemental Box *If the supplemental Box is not used, this sheet need not be included in the request.*

Continuation of Box No. IV

LUZZATTO, Edgar

LUZZATTO, Esther

HACKMEY, Michal

FUERST, Zadok

PYERNIK, Moshe

MANZUROLA, Emanuel

SERUYA, Yehuda

PRICE, Eyal

SHALEV, Ronit

HACKMEY, Miriam

P.O.Box 5352
Beer-Sheva 84 152
Israel

Box No. VI PRIORITY CLAIM Further priority claims are indicated in the Supplemental Box ☐

The priority of the following earlier application(s) is hereby claimed:

Country *(in which, of for which the application was filed)*	Filing Date *(day/month/year)*	Application No.	Office of filing *(only for regional or international application)*
item(1) IL	07 September 1998 (07.09.98)	126112	
item(2) IL	26 October 1998 (26.10.98)	126757	
item(3)			

Mark the following check-box if the certified copy of the earlier application is to be issued by the Office which for the purposes of the present international application is the receiving Office (a fee may be required):

☒ The receiving Office is hereby requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s): 1,2

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(If two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):* **ISA / EP**

Earlier Search *Fill in where a search (international, international-type or ther) by the International Searching Authority has already been carried out or requested and the Authority is now requested to base the international search, to the extent possible, on the results of that earlier search. Identify such search or request either by reference to the relevant application (or the translation thereof) or by reference to the search request:*

Country (or regional Office): Date *(day/month/year)*: Number:

Box No. VIII CHECK LIST

This international application contains the following number of sheets:

1. request	:	5 sheets
2. description	:	45 sheets
3. claims	:	9 sheets
4. abstract	:	1 sheets
5. drawings	:	14 sheets
Total	:	74 sheets

This International application is accompanied

1. ☒ separate signed power of attorney
2. ☒ copy of general power of attorney
3. ☐ statement explaining lack of signature
4. ☐ priority document(s) *identified in Box No. VI as item(s):*
5. ☒ fee calculation sheet
6. ☐ separate indications concerning deposited microorganisms
7. ☐ nucleotide and/or amino acid sequence listing (diskette)
8. ☐ other *(specify)*

Figure No. _____ of the drawings (if any) should accompany the abstract when it is published.

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request)

Michal Hackmey

For receiving Office use only

1. Date of actual receipt of the purported international application:
2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority specified by the applicant: **ISA/**
6. ☐ Transmittal of search copy delayed until search fee is paid

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

WO 00/14255
PCT/IL99/00483

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:
LUZZATTO, Kfir
Luzzatto & Luzzatto
P.O. Box 5352
84152 Beer-Sheva
ISRAËL

Date of mailing (day/month/year) 16 March 2000 (16.03.00)	
Applicant's or agent's file reference 7310-7311/WO/99	**IMPORTANT NOTICE**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/IL99/00483	06 September 1999 (06.09.99)	07 September 1998 (07.09.98)

Applicant YISSUM RESEARCH DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,CN,EP,JP,KP,KR,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CR,CU,CZ,DE,DK,DM,EA,EE,ES,FI,GB,GD,GE,GH,
   GM,HR,HU,ID,IL,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MN,MW,MX,NO,NZ,OA,PL,
   PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,UA,UG,UZ,VN,YU,ZA,ZW
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   16 March 2000 (16.03.00) under No. WO 00/14255

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer
J. Zahra

Telephone No. (41-22) 338.83.38

Continuation of Form PCT/IB/308

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

Date of mailing (day/month/year) 16 March 2000 (16.03.00)	IMPORTANT NOTICE
Applicant's or agent's file reference 7310-7311/WO/99	International application No. PCT/IL99/00483

The applicant is hereby notified that, at the time of establishment of this Notice, the time limit under Rule 46.1 for making amendments under Article 19 has not yet expired and the International Bureau had received neither such amendments nor a declaration that the applicant does not wish to make amendments.

IPEA/ EP

PCT

CHAPTER II

DEMAND

under Article 31 of the Patent Cooperation Treaty:
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty.

For International Preliminary Examining Authority use only

Identification of IPEA	Date of receipt of DEMAND

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION		Applicant's or agent's file reference 7310-7311/WO/99
International application No. PCT/IL99/00483	International filing date (day/month/year) 06 September 1999 (06.09.99)	(Earliest) Priority date (day/month/year) 07 September 1998 (07.09.98)

Title of invention

REGULATION OF GENE EXPRESSION THROUGH MANIPULATION OF mRNA SPLICING AND ITS USES

Box No. II APPLICANT(S)

Name and address:

YISSUM RESEARCH DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM
46 Jabotinsky Street
P.O. Box 4279
Jerusalem 91042
Israel

Telephone No.:

Facsimile No.:

Teleprinter No.:

State (i.e. country) of nationality: IL	State (i.e. country) of residence: IL

Name and address:

KAEMPFER, Raymond
18 Neve Shaanan Street
Jerusalem 93707
Israel

State (i.e. country) of nationality: IL	State (i.e. country) of residence: IL

Name and address:

OSMAN, Farhat
Sakhnin 20173
Israel

State (i.e. country) of nationality: IL	State (i.e. country) of residence: IL

☒ Further applicants are indicated on a continuation sheet.

International application No.
PCT/IL99/00483

Continuation of Box No. II APPLICANT(S)

If none of the following sub-boxes is used, this sheet is not to be included in the demand.

Name and address:

JARROUS, Nayef
Str. 304, Home #22
Shefaram 20200
Israel

State (i.e. country) of nationality: IL

State (i.e. country) of residence: IL

Name and address:

BEN-ASOULI, Yitzhak
Kfar Hanagid 206
76875
Israel

State (i.e. country) of nationality: IL

State (i.e. country) of residence: IL

Name and address:

State (i.e. country) of nationality:

State (i.e. country) of residence:

Name and address:

State (i.e. country) of nationality:

State (i.e. country) of residence:

☐ Further applicants are indicated on another continuation sheet.

International application No.
PCT/IL99/00483

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is ☒ agent ☐ common representative

and ☒ has been appointed earlier and represents the applicant(s) also for international preliminary examination.

☐ is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

☐ is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address:

LUZZATTO, Kfir; LUZZATTO, Edgar; LUZZATTO, Esther; HACKMEY, Michal; FUERST, Zadok; PYERNIK, Moshe; MANZUROLA, Emanuel; SERUYA, Yehuda; PRICE, Eyal; SHALEV, Ronit; HACKMEY, Miriam
LUZZATTO & LUZZATTO
P.O.Box 5352
Beer-Sheva 84 152
Israel

Telephone No.:
(972-7) 6497-871

Facsimile No.:
(972-7) 6497-125

Teleprinter No.:

☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. IV STATEMENT CONCERNING AMENDMENTS0

The applicant wishes the International Preliminary Examining Authority*

(i) ☒ to start the international preliminary examination on the basis of the international application as originally filed.

(ii) ☐ to take into account the amendments under Article 34 of

☐ the description (amendments attached).

☐ the claims (amendments attached).

☐ the drawings (amendments attached).

(iii) ☐ to take into account any amendments of the claims under Article 19 filed with the International Bureau (a copy is attached).

(iv) ☐ to disregard any amendments of the claims made under Article 19 and to consider them as reversed.

(v) ☐ to postpone the start of the international preliminary examination until the expiration of 20 months from the priority date unless that Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)).

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments to the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Box No. V ELECTION OF STATES

☒ The applicant hereby elects all eligible States except .....
.....
.....
.....

International application No.
PCT/IL99/00483

Box No. VI CHECK LIST

The demand is accompanied by the following documents for the purposes of international preliminary examination:			For international Preliminary Examining Authority use only: received	not received
1. amendments under Article 34				
description	:	sheets	☐	☐
claims	:	sheets	☐	☐
drawings	:	sheets	☐	☐
2. letter accompanying amendments under Article 34	:	sheets	☐	☐
3. copy of amendments under Article 19	:	sheets	☐	☐
4. copy of statement under Article 19	:	sheets	☐	☐
5. other *(specify)*:	:	sheets	☐	☐

The demand is also accompanied by the item(s) marked below:

1. ☒ separate signed power of attorney
2. ☒ copy of general power of attorney
3. ☐ statement explaining lack of signature
4. ☒ fee calculation sheet
5. ☒ other *(specify)*: notification of bank transfer

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Zadok Fuerst

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:
2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):
3. ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply. ☐ The applicant has been informed accordingly.
4. ☐ The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.
5. ☐ Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.

For International Bureau use only

Demand received from IPEA on: